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We propose to generate a novel inducible bitransgenic system to examine the effect of the expression of a target oncogene, the polyoma middle T antigen, on the onset of mammary epithelium hyperplasia. Using this inducible system, the specific expression of a transgene can be initiated by the administration of an external compound. To establish such a regulatable system, we have introduced the regulator and the inducible target separately into mouse embryos to generate transgenic mouse lines. To target the expression of the regulator in mammary glands, we have placed the regulator under the control of the MMTV-LTR. Transgenic lines of the regulator and the target, polyoma middle T antigen have been generated. These lines are currently being crossed to generate bitransgenic lines and the expression of the target oncogene will be induced by administration of progesterone antagonists. The regulated expression of polyoma middle T antigen in the mammary gland of bitransgenic mice will allow the investigation of the potential protective effects of avarian steroid hormones on the development of mammary tumors in response to specific oncogene expression. The success of this approach may have far-reaching effects on the future understanding of the mechanisms of oncogenesis in mammary epithelium.

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INTRODUCTION

Significance

Breast cancer continues to be a prevailing disease among women in the United States. Though numerous models for breast cancer have been developed, the etiology of the disease remains obscure. Transgenic mice technology has offered a means of successfully targeting oncogenes to the mammary glands to help better understand the role these oncogenes play in the development of breast cancer. However, the constitutive expression of the oncogene does not allow for the assessment of critical time points at which the mammary glands are most susceptible to oncogenesis. Thus, it is most imperative that an inducible system be established to turn genes on and off, rendering it feasible to define the role a candidate oncogene would play in mammary gland oncogenesis.

A novel inducible bitransgenic mice system is being generated to meet this goal. It is made up of two lines of mice. The first line of mice, the regulator mice, carries a chimeric transcription factor which is activated by an exogenous ligand, is targeted to the mammary gland via the MMTV promoter. This chimeric regulator consists of three functional domains: an activation domain from HSV-VP16, a Gal4 DNA binding domain and a modified version of the progesterone receptor ligand binding domain which is responsive to anti-progesterone (i.e. RU486) but not to progestins or other endogenous ligands). The second line of mice, the target mice, carries an oncogene under the control of four yeast transcription factor Gal4 binding sites (referred to as 17X4) where the regulator can bind to. Two minimal promoters have been chosen for the target genes namely, the E1B TATA and the TK promoter. When these transgenic lines are crossed to create the bitransgenic mice, the activated regulator can then bind to the 17X4 Gal4 recognition sequences upstream of the target oncogene and induce the expression of the target oncogene upon the administration of RU486. The expression of an oncogene in the bitransgenic mice can be induced by RU486 at specific windows during development and the effects of this oncogene on mammary gland oncogenesis can be monitored. With this system, one can also study the interaction between hormones and the target oncogene during mammary gland development.

To test the functionality of our inducible system, we first crossed our expressing regulator mouse to the 17X4 int-2/fgf-3 target mouse generated by Ornitz [1]. Our justification lies on the fact that the 17X4 int-2/fgf-3 target mice can be activated by transcription factors harboring the Gal4 DBD and has been shown to induce hyperplasia in the mammary gland [1]. Having verified our regulator system, we will use the polyoma middle T antigen (PyT) as a target oncogene in our breast cancer model. PyT is a potent oncogene which has been shown to cause multi-focal tumors in the mammary glands of female mice [2]. This target will be regulated by our transactivator, which will be targeted for expression in the mammary glands. Our goal will be to study the effects of regulated PyT expression in the mammary gland in an effort to better understand the mechanism involved. The success of our system will have a far-reaching impact in the understanding and in the ultimate goal of combating breast cancer.

BODY

Summary of previous work

Our previous work documented that we had 3 lines of regulator expressing mice. The MBGH line expressed the regulator at high levels while the other BHMM and MVPC regulator lines express the regulator at low levels as analyzed by Northern analyses. We also mentioned that we were breeding up the MBGH line to begin crosses with the 17X4 int-2/fgf-3 target mice obtained from Dr. Phil Leder.

A preliminary profile of PyT target gene expression in different tissues from the two 17X4-PyT target lines 5988 and 5989 was also presented. We were testing these target lines to see if they meet the criteria ascribed (i.e. target is silent in the absence of the transactivator).

(Work from Oct. 1996 to Oct. 1997)

Summary of analyses of the 17X4-PyT targets

To ensure that the target line is silent, we analyzed the tissue-profile of target gene expression in both male and female target mice by the more sensitive RT-PCR method. In the initial breeding stages, we had fewer females and so we begun to analyze the expression of the male target mice first. The results summarized in Table 1 were derived from male target mice. In the 5988 line, no expression of the target was seen in the tested tissues. In the 5989 line, we observed low levels of target expression in the brain.

The RT-PCR analyses of the tissue-profile of target expression from day 10 lactating female PyT target mice are shown in Table 2. Analysis of the female 5989 target line showed that the expression of the target was detected only in the brain. In the 5988 female target line, expression of the target was seen in the liver, heart, brain and salivary gland. We are currently expanding the lines to use them for breeding with a functional regulator line to generate bitransgenic mice for our studies.

Since we had easy access to the 17X4 int-2/fgf-3 target mice which had also been proven to be inducible [1], we had decided to devote our efforts in testing the functionality of our regulators with the 17X4 int-2/fgf-3 target mice while the 17X4 PyT target mice were breeding. Once we have found a functional regulator, we can then focus on using these targets as a model for PyT-induced breast cancer.

Profile of regulator expression during mammary gland development

It is well documented that the expression of MMTV-driven transgenes peak at lactation.

MMTV-driven transgenes are expressed constitutively; being usually low at the virgin stage, increases through pregnancy, reaches its maximum levels at lactation and falls during involution to levels similar to the virgin stage. Other factors can affect this profile of expression and they include integration and chromosomal effects.

To verify this observation, we have begun to analyze the expression of the MMTV-regulator from the MBGH 6219 line (highest regulator expressor) during the virgin (vir), pregnant (preg) and lactation (lac) stages by Northern analyses. We failed to detect the expression of the regulator during the virgin stage but the levels of the regulator are relatively high during pregnancy and lactation after normalization to a duplicate blot probed with actin (**FIGURE 1**). No regulator expression was detected in the wildtype virgin (wt vir) or wildtype lactating (wt lac) controls. Thus, my results are in agreement with the published profile of expression of MMTV-driven transgenes.

In order for the regulator to be a strong transactivator, its levels should at least be moderate and be detectable by Northern analysis. This will ensure that enough regulator protein can be made and when activated by RU486 can then induce target gene expression in bitransgenic mice.

Generation of bitransgenic mice

One of the difficulties attributed to working on the mammary gland is to obtain sufficient female mice for analyses. This difficulty is further compounded by the need to obtain bitransgenic female mice (i.e. mice harboring both the regulator and target transgene) and the reduced probability hampers the efficiency of our analyses.

To alleviate this problem, we have initiated multiple matings among MBGH regulator and 17X4 int-2/fgf-3 target mice to increase the number of female bitransgenic mice for our studies. This justification is substantiated by a report from Phil Leder's lab that a highly expressing Gal4 transactivator is able to significantly induce the expression of the 17X4 int-2/fgf-3 target gene in bitransgenics [1]. Hence, the time needed to perform an induction experiment will be determined by how good a certain breeding pair is and this can take quite a considerable period of time.

Based on the finding that both the BHMM 7386 and MVPC 6921 lines expressed the regulator at low levels by Northern analyses, we have decided to focus our efforts on the highly expressing MBGH 6219 regulator line which preferentially expresses the regulator at a high level in the mammary gland.

Genotyping of mice

Progeny mice born from a cross between the MBGH regulator and the 17X4 int-2/fgf-3 target mice were subjected to genotyping by PCR. Since the 17X4 int-2/fgf-3 target mice are

homozygotes, a cross between this line and the regulator line should yield only target and bitransgenic mice.

Induction of int-2/fgf-3 target

Since the expression of MMTV-driven transgenes peak at lactation, we have chosen this as a first time point for our induction studies. Female bitransgenic mice were mated with a virile stud male and allowed to progress through lactation. Upon parturition (considered to be day 1 of lactation), 250μg/Kg bodyweight of RU486 dissolved in sesame oil or sesame oil vehicle were given to the respective experimental and control mice on alternate days (i.e. day 1, 3, 5 & 6) for a week. Twelve hours following the last injection of RU486, mammary biopsies were performed to obtain RNA for analysis.

Since the pregnancy period also demonstrates a high level of regulator expression, we also performed induction studies for this time point. A dose of 250μg/Kg bodyweight of RU486 and sesame oil vehicle were given to respective experimental and control mice at day 15 of pregnancy on alternate days (i.e. day 15, 17, 19 of pregnancy). Similarly, twelve hours after the last RU486 injection, mammary biopsies were performed to obtain RNA to analyze for target gene induction.

Results of induction studies

RNA was isolated from the mammary glands of control and experimental and Northern analyses were performed with 20μg of total RNA. The RNAs were probed for both regulator and int-2/fgf-3 target genes in separate blots.

FIGURE 2 showed no apparent change in regulator expression in both the control and RU486-treated animals. This result implied that the level of RU486 administered did not perturb the activity of any endogenous progesterone or glucocorticoid receptors and hence the MMTV promoter.

It was also evident that the expression of int-2/fgf-3 was not induced under the experimental conditions (**FIGURE 3**). A positive control containing RNA from the lung (fgf-3/int-2 +) of an inducible bitransgenic animal obtained from Dr. Franco DeMayo's lab indicated that the Northern analysis was working properly.

To exclude the possibility that the levels of int-2/fgf-3 expression was too low to be detected by Northern analysis, we used a more sensitive ribonuclease protection assay (RPA) to examine for int-2/fgf-3 induction (**FIGURE 4**). We still failed to see any int-2/fgf-3 induction in both our control and experimental samples even though the int-2/fgf-3 protected band was evident in the positive control. The 28S riboprobe was used for normalization.

These results indicated that our regulator though expressed highly did not activate the int-2/fgf-3 target gene under both pregnancy and lactation time points. Our failure to induce the int-2 target gene prompted us to examine the possible causes.

We compared the MBGH regulator mRNA to the lung regulator message by Northern analysis and found that the MBGH message was significantly larger than the mRNA of a functional lung regulator obtained from Dr. Franco DeMayo (**FIGURE 1**). We were able to exclude intron inclusion and we ascribed this anomaly most likely to rearrangement after integration of the transgene into the mouse genome.

Construction of an improved regulator (HMMB)

While we were analyzing for the induction potential of the MBGH regulator, we had begun to generate an improved HMMB regulator. The regulator is shown schematically (**FIGURE 5**). We had cloned the more potent form of the regulator which had been shown to possess both a higher affinity for RU486 and a higher transactivation potential [3] into the rabbit betaglobin gene fragment (KCR) containing a strong bovine growth hormone polyadenylation signal (bGHpA) under the control of the MMTV promoter. To minimize the effects of integration, we inserted a 2.4 Kb dual-copy fragment of the chickenβ-globin gene insulator (HS4) [4] at the 5' end of our transgenic construct.

To minimize the expensive cost of generating transgenic mice, we verified the functionality of the HMMB regulator with transfection experiments performed in T47D human breast cancer cells. As shown in the graph (**FIGURE 6**), the HMMB regulator was able to transactivate the 17X4-TATA-luciferase reporter only when RU486 was given.

Genotypic analysis for HMMB founders

Having substantiated the transactivation potential of the HMMB regulator in the presence of RU486 in transfections, we linearized the construct and purified it for one-cell microinjection. As shown in the Southern analyses (**FIGURE 7A & 7B**) with the appropriate microinjection fragment controls (0, 1, 10 copies) and a positive MVPC control, we obtained 6 founders (denoted by *) harboring the HMMB transgene out of 8 mice born. These results are also summarized in **TABLE 3**. We bred the founders to expand the different lines to enable expression studies to be performed.

To determine the passage of the regulator transgene from the founders, Southern analyses on genomic DNA digested by BglII were performed. As summarized in **TABLE 4**, the two founder lines 9476 and 9480 were mosaics and they did not pass the transgene on to their progeny. One of the lines, 9477, was a multiple integrant and these different integration sites segregated into 3 groups. One was the parental multiple integrant pattern (*) and the other two were different segregated patterns (# and +). This is depicted in the Southern analysis shown in **FIGURE 8**. Analyses of the remaining lines 9475, 9478 and 9481 showed that they all passed the transgene in the normal Mendelian fashion.

Expression of HMMB regulator in mammary gland

From our previous experience, we have concluded that for a regulator to be functional, a number of criteria will have to be met. The regulator has to be expressed at a level detected at least moderately by Northern analysis, the mRNA size has to be correct and the message has to be expressed predominantly in the mammary gland. Towards this goal of obtaining functional and expressing regulator lines, mammary biopsies were performed on day 10 lactating regulator females to obtain RNA for regulator expression studies. Since 9476 and 9480 were male mosaics, expression studies could not be performed on them. As depicted in the Northern (**FIGURE 9**), the 9478 line expressed a reasonable level of correct transcript, the 9475 line expressed a low but detectable level while the 9477 nor the 9481 lines showed no expression. A duplicate blot was probed with cyclophilin to normalize loading. Based on these results, we focused our studies on the HMMB 9478 expressing line.

Generation of bitransgenic mice

To expedite the generation of bitransgenic female mice for induction studies, we had crossed the HMMB 9478 female founder to a 17X4 int-2/fgf-3 target male. Five progeny mice were born from this cross and as demonstrated by the respective Southern analyses for regulator and int-2/fgf-3 target genes (**FIGURE 10**), one female bitransgenic mouse (#392) was obtained. We mated this bitransgenic female #392 to a wildtype stud male to allow us to assess int-2/fgf-3 induction in the lactating mammary gland.

Induction of int-2/fgf-3 in bitransgenic #392 mouse

The #392 bitransgenic female developed normally through pregnancy. Upon parturition, we noticed that all her pups were dead and they do not contain milk in their stomach. This fact is compatible with the finding by Ornitz that the overexpression of int-2/fgf-3 in the mammary glands resulted in failure of lactation [1]. Gross analysis of this mouse (d3pp) revealed numerous mammary gland tumors (Mgt) as indicated by arrows (**FIGURE 11, panel A**). A couple of wart-like structures (WLS) were also seen to project out from her nipples. Gentle squeezing of the mammary glands did not reveal any milky exudate. A conspicuous salivary gland tumor (SGt) on her left cheek was also present.

Based on these observations, we surmised that we probably had leaky expression of the int-2/fgf-3 target gene in the absence of RU486 administration. It is known that the 17X4 int-2/fgf-3 target mice are silent in the absence of any transactivator [1] and the leakiness is probably due to the regulator activating the target gene.

RU486 studies on the #392 bitransgenic mouse

To determine if an upregulation of int-2/fgf-3 gene expression was observed in the presence of RU486, we had decided to take mammary gland biopsies before and after RU486 addition in the same animal to perform molecular and histological analyses.

On day 3 after the #392 bitransgenic female had given birth, we obtained a right 4th mammary gland wholemount from her. A tumors mass attached itself to the bodywall and seemed to originate from the right 4th mammary gland. We resected that tumor and also masses from the right 5th mammary glands for RNA analysis. The wart-like structures (WLS) that extended from the nipples of her left 2nd and 4th mammary glands (**FIGURE 11, panel A**) were resected and processed for fixation. Since we only had one female bitransgenic mouse, we used these samples as control (i.e. before RU486 addition).

We then gave RU486 at a dose of 250µg/Kg bodyweight in sesame oil by intraperitoneal injection for 3 consecutive days (day 3, 4 & 5) and sacrificed the animal 12 hours following the last injection. The left 3rd and 4th mammary glands were processed for wholemount analysis and the rest of the mammary glands were used for RNA analysis. The left salivary gland tumor was also resected for both RNA and histology. A normal salivary gland on the right side was processed for both RNA and histology. To ascertain if the regulator was also expressed in other organs, the liver, spleen and lungs were also processed for RNA analysis. All these tissue samples fall into the experimental group (i.e. with RU486 administration).

Histology of the tumors

Prior to RU486 administration

Wholemounts:

We processed the right 4th mammary gland wholemount by carnoy's fixation and carmine staining and performed histological analysis after clearance by xylene. As depicted in **FIGURE 12**, we saw massive compaction of epithelial cells consistent with the lactation period (**panel D**). Since this female was only 8 weeks old at the time of pregnancy, her mammary tree did not cover the whole of the fat pad (FP). We also observed a possible tumor mass (*) that appeared as an indentation (**panels C & G**). The lymph node (#) was indicated for orientation (**panels B, C, E & F**).

To allow for a better appreciation of the cellular morphology of the mammary gland, we resected the tumor mass from the wholemount and embedded these tissues separately in paraffin for sectioning at 6µm thickness. The tumor section (**R4MG tumor**) showed massive collagen infiltration around the hyperplastic (hyp) mammary ducts (md) indicating that it was relatively high grade (**FIGURE 13, panel C**). The collagen also appeared to deform the mammary ducts. Prominent blood vessels (bv) could be seen to penetrate the densely packed mammary epithelia (**panels A & B**). No normal morphology was observed.

Analysis of the wholemount section (**R4MG-WM**) in **FIGURE 13** revealed various

grades of hyperplastic growth (**panels D & E**). Normal morphology was evident in the form of adipose tissue (ad) around mammary ducts (**panel D & E**). However, massive collagen infiltration around compacted mammary ducts were also present (**panel F**). The lymph node (LN) is as indicated (**panel D**). These heterologous manifestations implied that the penetrance of the regulator transgene was not entirely homogenous.

Wart-like structure sections:

Examination of the wart-like structures (WLS) revealed that they were outgrowths from the mammary gland (**FIGURE 14**). The diagnostic epithelial architecture of the skin was evident at the outer boundary of the structures (**panels B, C, E & F**). Internally, mammary ductile structures (md) together with numerous blood vessels (bv) were observed (**panels B & C**). Once again, varying grades of hyperplastic growth (hyp) were salient (**panels C & F**). The presence of mammary ducts ruled out the possibility that these structures were papillomas.

After RU486 administration

Wholemounts:

The gross pathology of the bitransgenic mouse after RU486 administration for 3 days at a dose of 250 μg/Kg bodyweight is shown in **FIGURE 11, panel B**. We could still feel lumps in her mammary glands and the salivary gland tumor was still evident. To get a better appreciation of the effects of RU486 administration on our bitransgenic mouse at the histological level, we processed the left 3rd (**L3MG**) and 4th (**L4MG**) mammary glands for wholemount analysis as depicted in **FIGURE 15**. Since the pups had died and no suckling had taken place, the mammary epithelia would have proceeded to involute. Thus, we found that the mammary gland displayed more regions that contain the fat pad (FP) indicating that some involution could have taken place when compared to the wholemount at day 3 lactation (R4MG) in **FIGURE 12**. . However, regions of hyperplasia (hyp) characteristic of the dense packing epithelia cells of the lactation phase were still evident. It was also evident that the terminal end buds (teb) did not cover the whole fat pad as the animal was only 8 weeks old at time of sacrifice (**panels B & D**).

Wholemount sections:

Paraffin sections of the left 3rd (**L3MG**) and 4th (**L4MG**) mammary gland showed a higher proportion of normal morphology indicating that regression had taken place (**FIGURE 16**) when compared to the right 4th mammary gland section (**FIGURE 13**). Regions containing normal adipose tissues (ad) and ducts (md) can be observed (**panels A-E**). However, varying grades of hyperplastic growth (hyp) with numerous blood vessels (bv) were still evident (**panels A, B, D & E**). Massive collagen infiltrated regions (**panel C**) were still clearly demarcated indicating a similar picture as above (**Figure 13, panels C & F**).

These histological analyses convinced us that the hyperplastic and tumorigenic growth were present. To ascertain whether these manifestations were the result of regulator induced int-2/fgf-3 expression, we performed Northern analyses.

Molecular analysis

Regulator expression:

We processed RNA from the mammary gland and salivary gland tumors, liver, spleen and a normal salivary gland for northern analysis. We probed separate Northern blots for regulator and int-2/fgf-3 target gene expression. In **FIGURE 17**, we observed that the regulator was expressed fairly highly in the mammary gland and salivary gland tumors but not in the liver, spleen or the normal salivary gland. Once again, the levels of the regulator did not change with RU486 addition.

int-2/fgf-3 expression:

The expression of the int-2/fgf-3 target gene mirrored that of the regulator indicating that the regulator was responsible for activating the int-2/fgf-3 gene (**FIGURE 18**). Judging from the GAPDH and ribosomal RNA normalization, we could see a modest induction of the int-2/fgf-3 target gene with RU486 administration. However, our system was not silent in the absence of RU486. Based on the finding that neither the 9478 female founder nor any of the int-2/fgf-3 target females displayed any palpable tumors during parturition and the fact that tumors had arisen only in the bitransgenic female, we were confident that these tumors were the result of regulator-activated int-2/fgf-3 gene expression.

Endogenous MMTV cooperation

The kinetics of tumor formation by int-2/fgf-3 expression in the mammary gland is not compatible with that reported in the literature [1, 5]. Ectopic expression of int-2/fgf-3 in the mammary gland generated hyperplasia and tumors form only after a prolonged period of time in the endogenous MMTV-free fvb strain of mice. Our rapid onset of tumor formation was indicative of possible cooperation event(s). One possibility was that our mice strain contained an endogenous MMTV infection. This seemed to be the case as our mice had the C3H background, which carried endogenous MMTV infections.

To ascertain this, Northern analysis performed with RNAs isolated from the various tissues of the bitransgenic mouse using a MMTVC3H Orf probe obtained from Dr. Janet Butel showed that indeed, an endogenous MMTV C3H infection was present. In particular, the mammary gland and salivary gland tumors showed high levels of 3 specific mRNAs corresponding to the MMTVgRNA, MMTV env and MMTVLTR as depicted in **FIGURE 19**. As shown in **FIGURE 20**, analysis of the same samples with the MMTV env probe revealed two specific mRNAs with a profile of expression similar to that of the Orf probe. These results indicated that we had endogenous MMTV infection and that the MMTVLTRs might have activated a gene(s) that could collaborate with int-2/fgf-3.

Possible candidate collaborating genes include int-1/wnt-1 [6, 7] and wnt-10b [8]. To assess the possibility that either of the two genes could collaborate with int-2/fgf-3, we are currently performing Northern analyses to address these questions.

ONGOING EXPERIMENTS

Expand the HMMB 9478 line

We are currently expanding the 9478 line to allow us to perform more induction experiments. In particular, this would allow us to test the reproducibility of the rapid tumorigenesis event observed in the #392 bitransgenic female and to ensure that the event is not fortuitous to begin with. Our recent breeding yielded 14 pups of which 6 pups (denoted by *) received the regulator transgene from the HMMB 9478 founder as documented by Southern analysis (**FIGURE 21**). It was imperative that we perform the Southern analysis to ensure that the founder was not a multiple integrant and that the progeny display the same pattern of integration as the founder. Once we have sufficient regulator mice, we would also perform a study on the expression profile of the regulator in different tissues. Our preliminary results indicated that the expression of the regulator is relatively specific (**FIGURE 17**).

Induction studies at different mammary gland developmental stages

It is evident that our HMMB 9478 line is expressing a regulator that is able to transactivate the int-2/fgf-3 target in the #392 bitransgenic mouse. While we were not able to obtain tight regulation during the lactation time points, it is possible that we will be able to get a tighter regulation during different stages in the mammary gland development (i.e. virgin and late involution stages) when we have lower levels of regulator expression. We will perform the induction studies in female bitransgenic mice at these stages when we have obtained sufficient animals.

Utilization of the 17X4-PyT target

With the generation of more 17X4-PyT mice, we should be able to test if our functional regulator could activate these targets. Once we have ascertained that our regulator can induce the 17X4 PyT mice, we can proceed with experiments aimed at deciphering the consequences of regulated PyT ectopic expression at different stages of mammary gland development. We could also take advantage of the PyT system for a metastasis model. The results of these studies will greatly help us to better understand the mechanism of PyT induced tumorigenesis.

CONCLUSIONS

The highly expressing MBGH regulator mouse is not functional and based on the results of our analyses; we can specify more stringent requirements that a regulator line must meet to ensure success. The regulator mouse has to express the regulator at a level detectable by Northern analysis and it is very crucial that the size of the message is correct. Failure to meet any of the two above criteria severely lowers the chance that a particular regulator line will be successful in inducing target gene expression.

The HMMB 9478 line expressed the regulator at a moderate to high level detectable by Northern analysis and the mRNA is of the correct size. Based on analyses on the bitransgenic mouse #392 (that harbors this regulator and the fgf-3/int-2 target), we know that the regulator is functional and is able to activate the target gene. Our present study indicated that we do observe a modest induction of the int-2/fgf-3 target in our bitransgenic female mammary glands after RU486 administration. However, the regulator appears to activate the target gene in the absence of RU486 administration. A likely explanation for this is that this regulator contains the dimerization domain of the Gal4 DBD in addition to the RU486-dependent one in the PRLBD and when produced in sufficient amounts, the former could dimerize and bind to DNA to transactivate the target gene even when RU486 is absent. To obtain tighter regulation, we will perform the induction studies at the virgin stage and late involution stages in the bitransgenic mice. At such stages, the lower levels of regulator expression could potentially enhance the usefulness of our inducible system.

The ultimate regulator and target pair would require extensive testing to find the perfect match. Other factors that affect the ability of a target to be induced include integration and epistatic chromosomal effects. The selection of a silent target does not necessary translate into a good target as this line could just not be activated even by the most potent regulator. The 17X4 int-2/fgf-3 target mice are selected to be easily activated and the selection of a target line that is more difficult to activate could be the key. With sufficient 17X4 PyT targets generated, we can proceed to cross them to our functional regulator line to generate bitransgenic mice for our induction studies. The ability of our inducible system to regulate the expression of PyT during mammary development will provide a useful model for mammary carcinogenesis. The far-reaching impact of our study will greatly aid the understanding of breast cancer and also provide means to ultimately design better therapeutic agents.

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Profile of PyT gene expression in male target mice

	Tissue	Expression
Male 5989	Lung	No
	Testis	No
	UG Tract (urethra/prostate)	No
	Brain	Yes
	Salivary gland	No
Male 5988	Lung	No
	Testis	No
	UG Tract (urethra/prostate)	No
	Spleen	No
	Liver	No
	Brain	No
	Heart	No

TABLE 1

Profile of PyT gene expression in female target mice

	Tissue	Expression
Female 5989	Lung	No
	Mammary gland	No
	Liver	No
	Heart	No
	Brain	Low
	Salivary gland	No
	Spleen	No
Female 5988	Lung	No
	Mammary gland	No
	Liver	Low
	Heart	Yes
	Brain	Low
	Salivary gland	Low
	Spleen	No

TABLE 2

Profile of regulator expression during mammary gland development

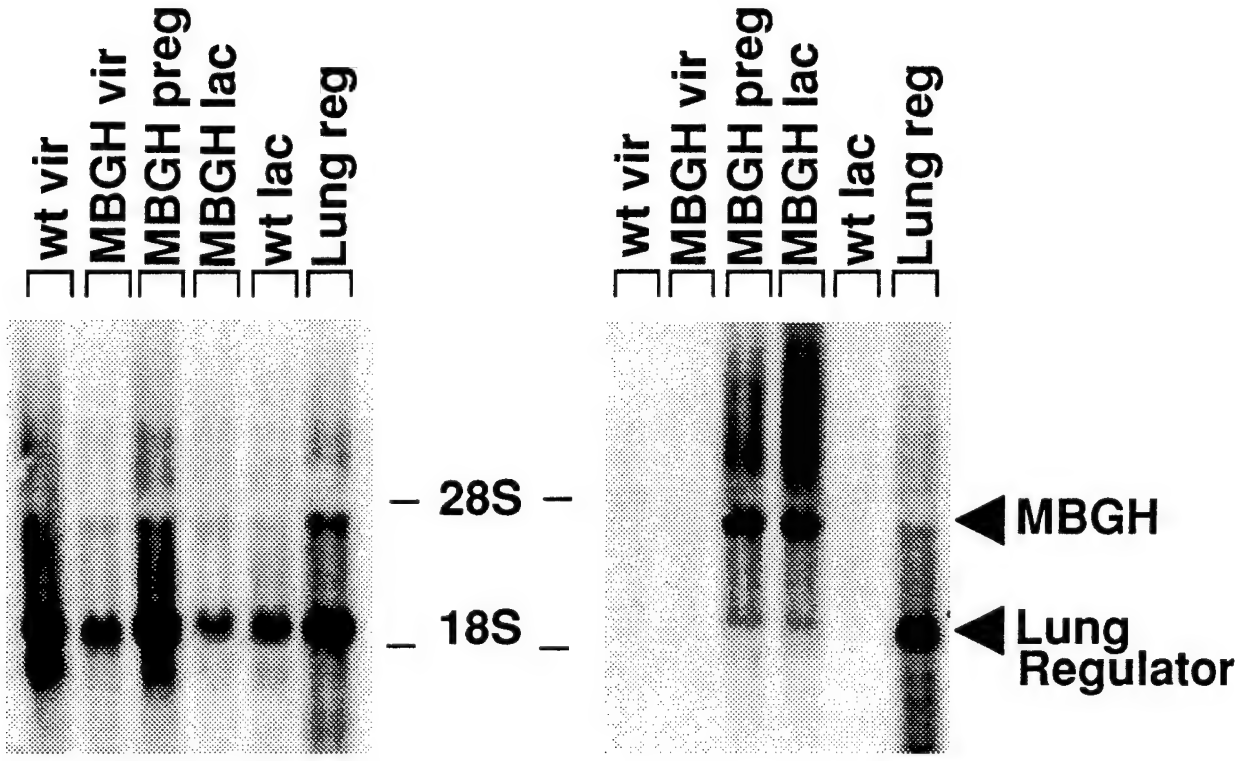


FIGURE 1

Northern analysis of regulator expression in bitransgenic mice

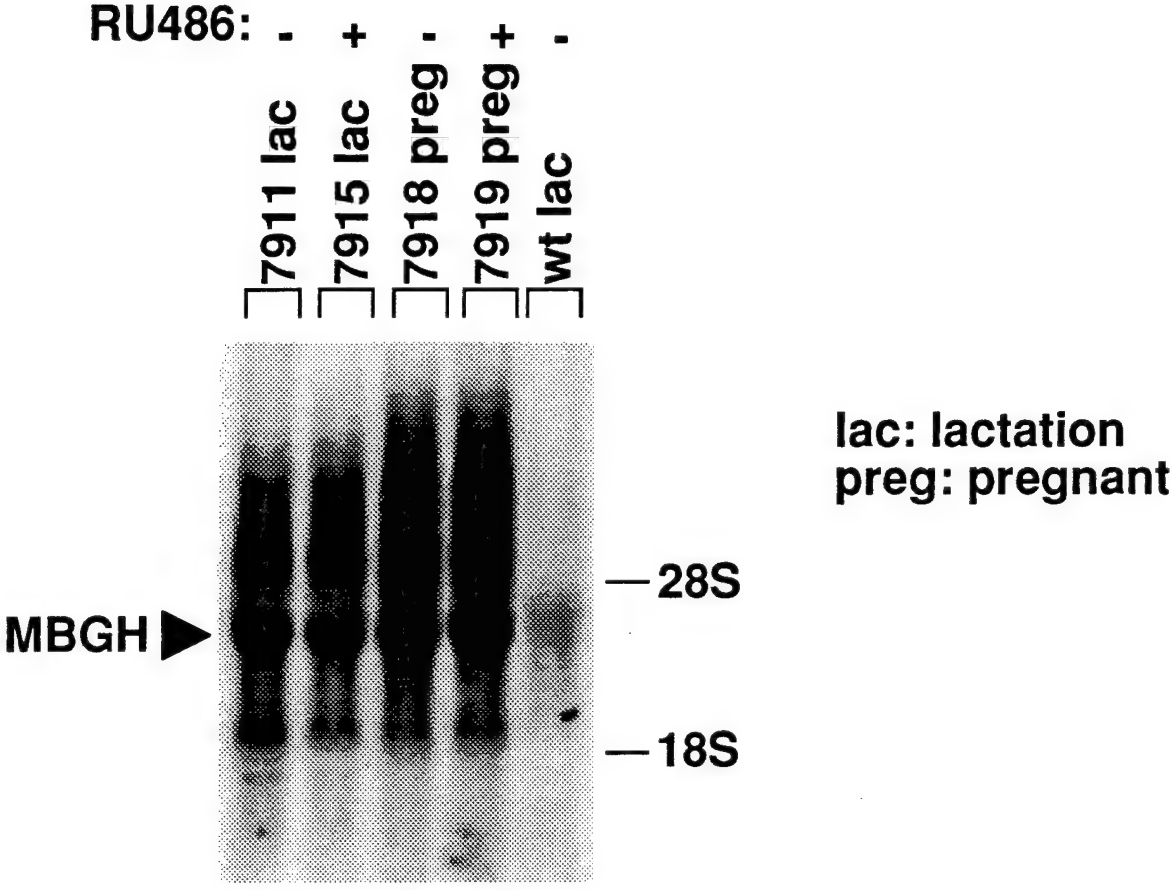


FIGURE 2

Northern analysis of Bitransgenic mice MG RNA for fgf-3/int-2 target gene induction

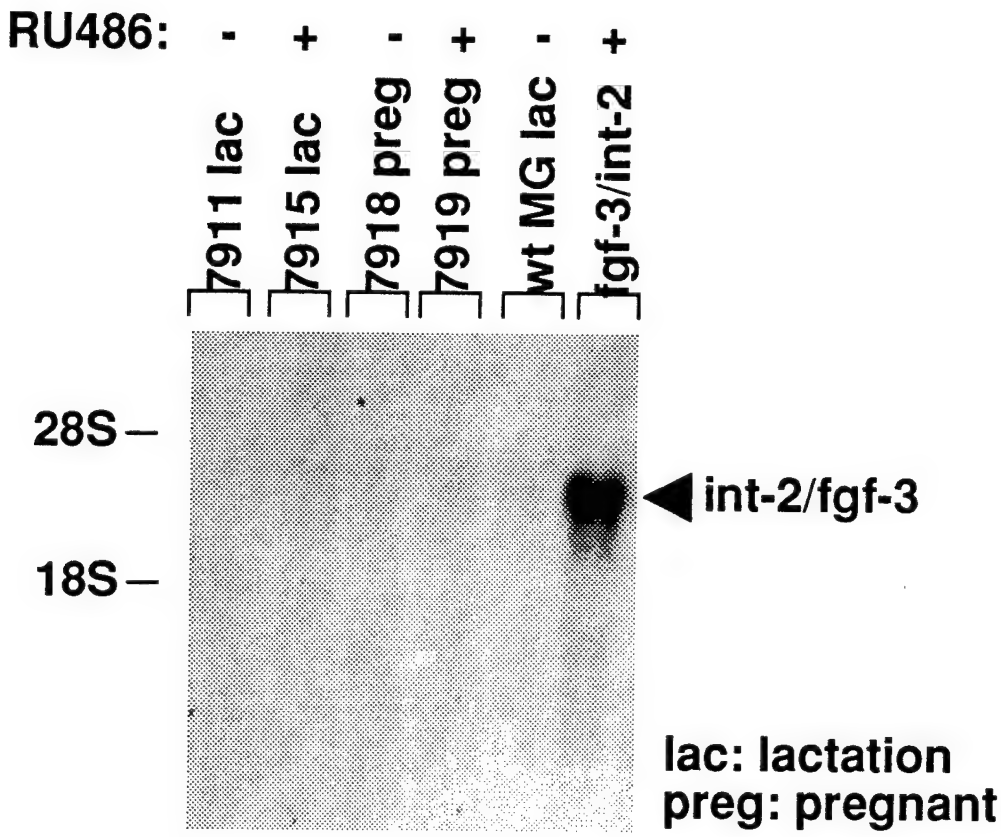
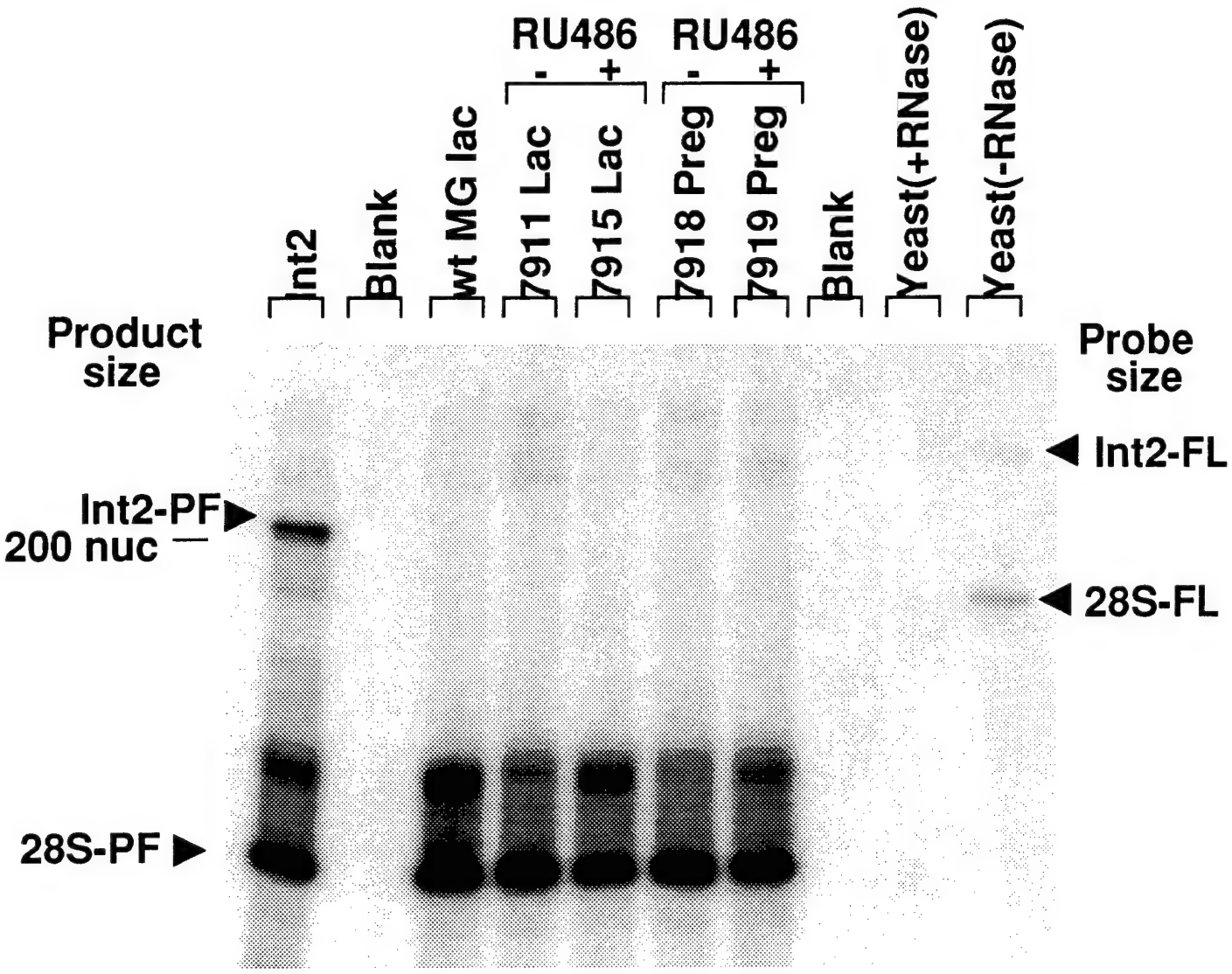


FIGURE 3

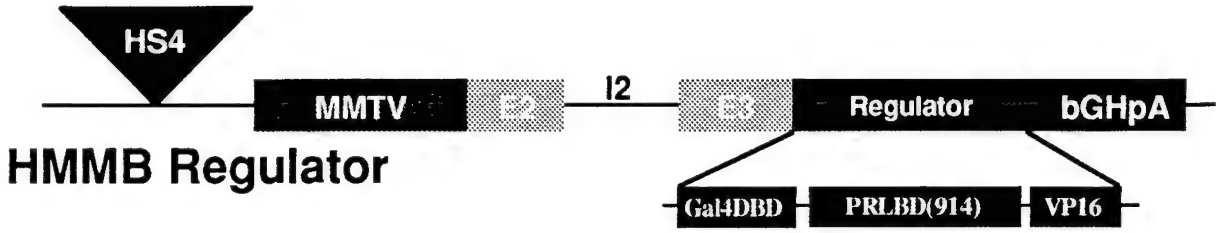
Ribonuclase protection analysis of bitransgenic mice RNA for int-2/fgf-3 target gene induction



lac: lactation
preg: pregnant

FIGURE 4

Schematic representaion of the HMMB regulator



HMMB Regulator

HS4: 2.4 kb insulator fragment

KCR: Exon 2 (E2), Intron 2 (I2) and Exon 3 (E3) of the rabbit betaglobin gene fragment

Gal4DBD: Gal4 DNA binding domain

PRLBD(914): progesterone ligand binding domain responsive only to RU486 activation (aa641 to aa914)

VP16: activation domain from HSV-VP16

bGHpA: bovine growth hormone poly A signal

FIGURE 5

Transactivation potential of the HMMB regulator

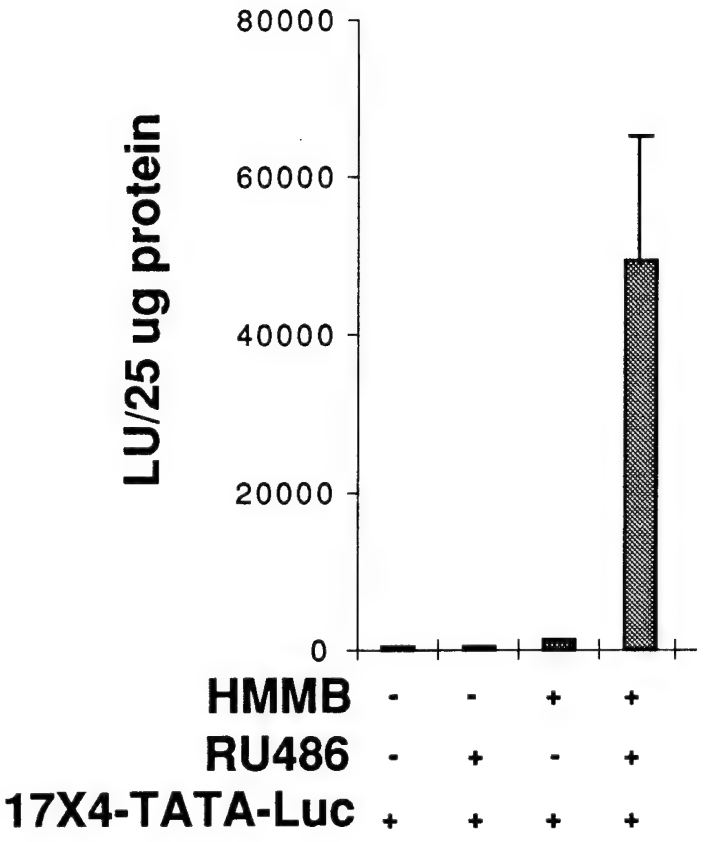


FIGURE 6

Summary of HMMB genotypic analysis

Mom#	Eartag#	Sex	Transgenic	Approx copy#
658	9474	F	No	NA
	9475	F	Yes	50
	9476	M	Yes	1
660	9477	F	Yes	~5
	9478	F	Yes	~5
	9479	M	No	NA
	9480	M	Yes	~10
	9481	M	Yes	~10

TABLE 3

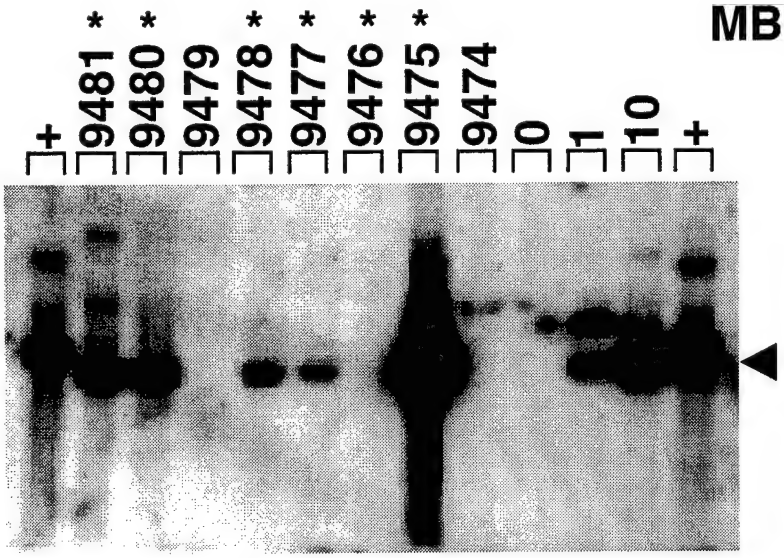


FIGURE 7A

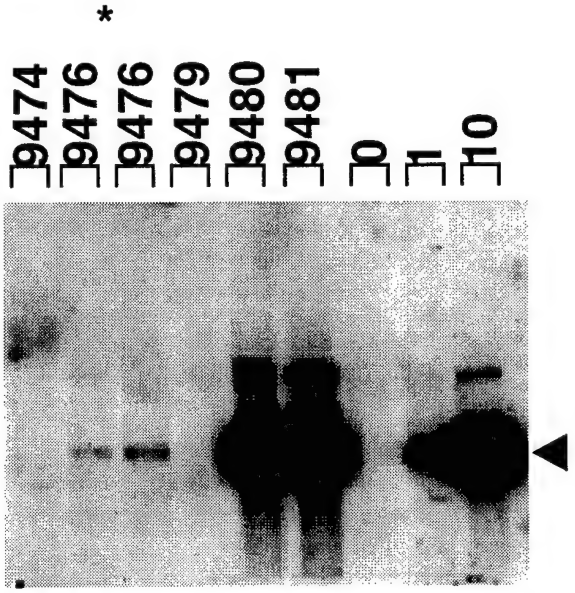


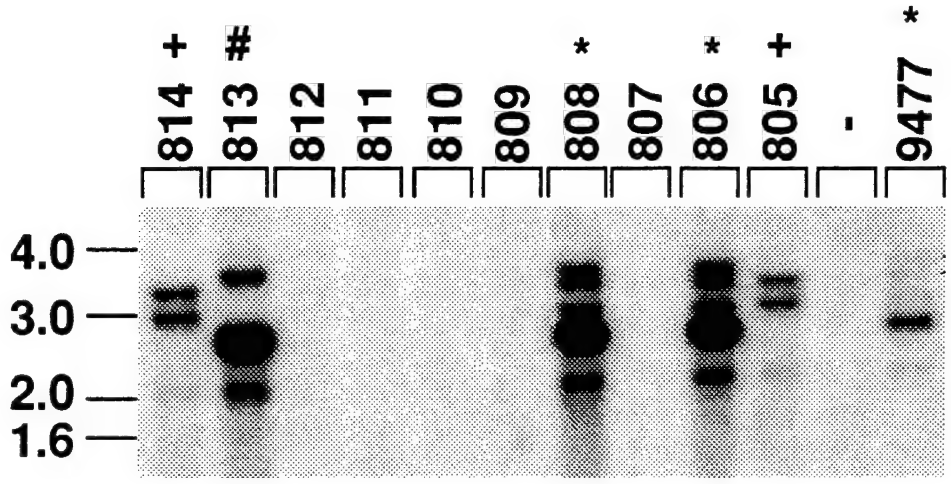
FIGURE 7B

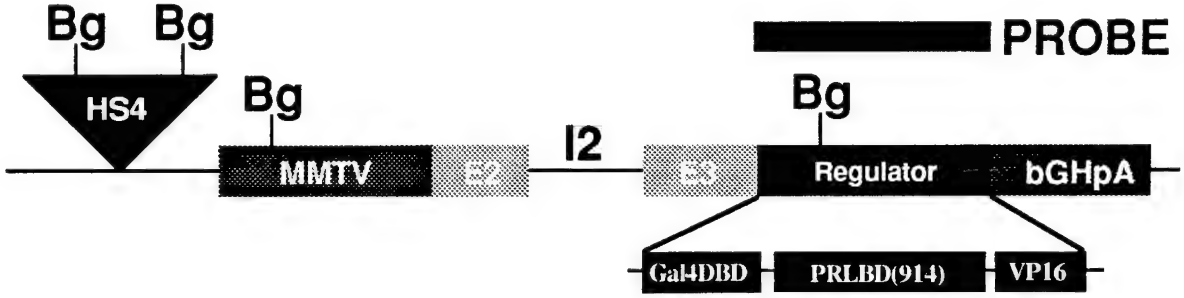
Summary of HMMB mice analyses

Founder	Sex	TG copy #	Transgene passage	MG expression
9475	F	50	Mendelian	Very low
9476	M	1	Mosaic	ND
9477	F	~5	Multiple Integ	No
9478	F	~5	Mendelian	Med
9480	M	~10	Mosaic	ND
9481	M	~10	Mendelian	ND

TABLE 4

Southern analysis of the 9477 multiple integrant line





* Parental pattern
# Segregated pattern 1
+ segregated pattern 2
Bg: BglII

FIGURE 8

Northern analysis of lactacting MG RNA for regulator gene expression

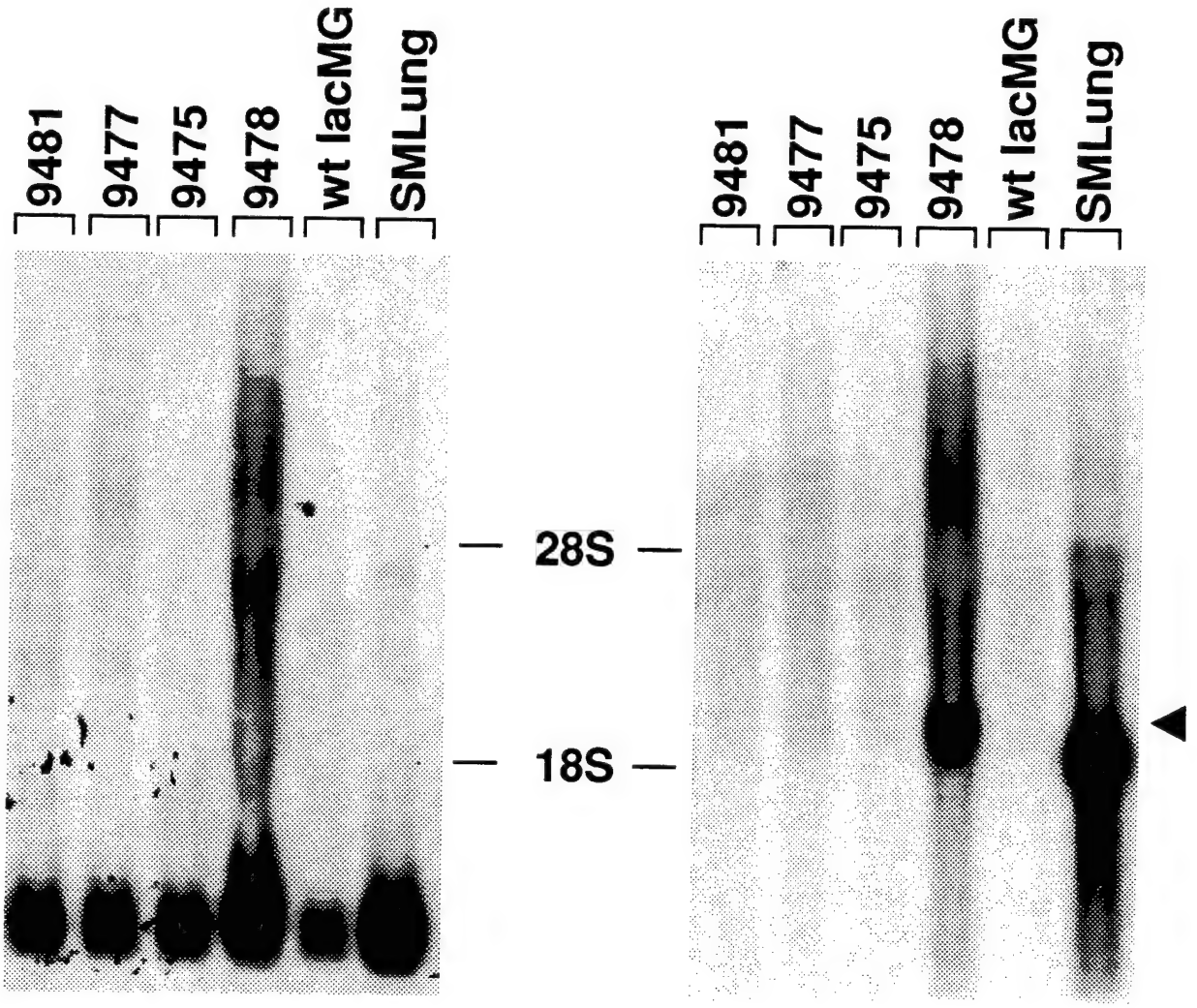
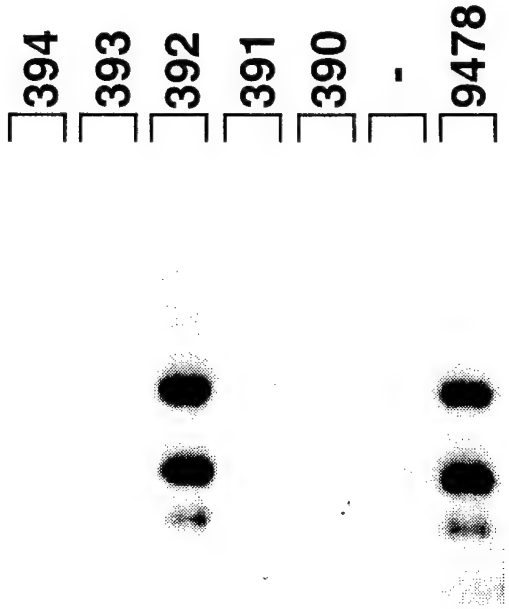
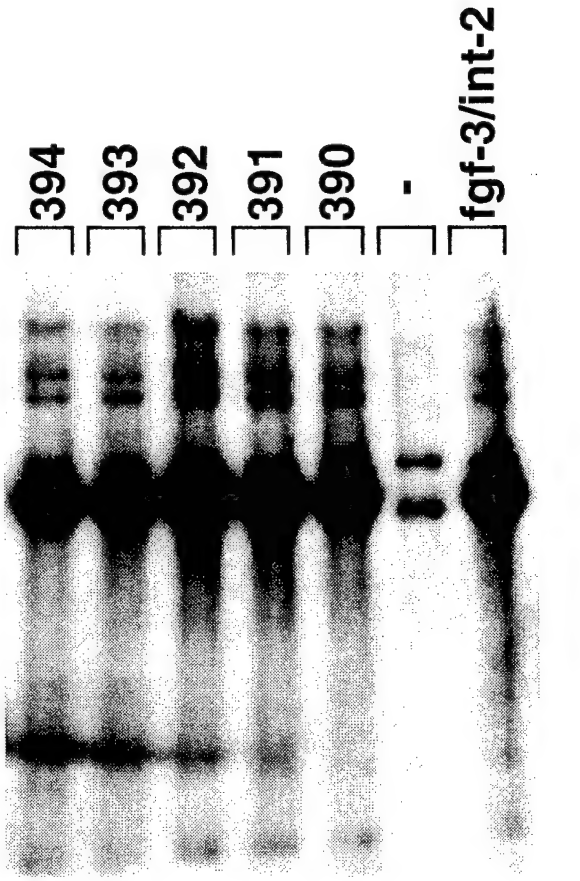


FIGURE 9

Southern analysis of mice tail DNA of progeny from a cross between HMMB 9478 and fgf-3/int-2 target mice



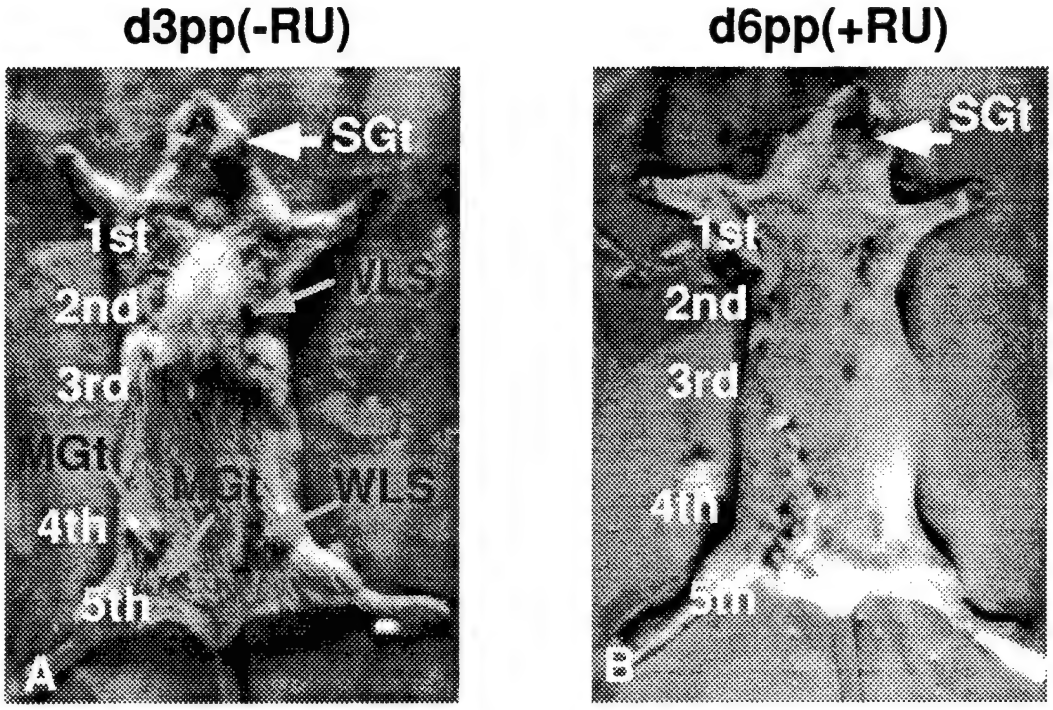
HMMB regulator



fgf-3/int-2 target

FIGURE 10

Gross pathology of bigenic lactating female mouse



d3pp: day 3 postpartum
d6pp: day 6 postpartum

FIGURE 11

Wholemount analysis of #392 bitransgenic mouse mammary gland before RU486 administration

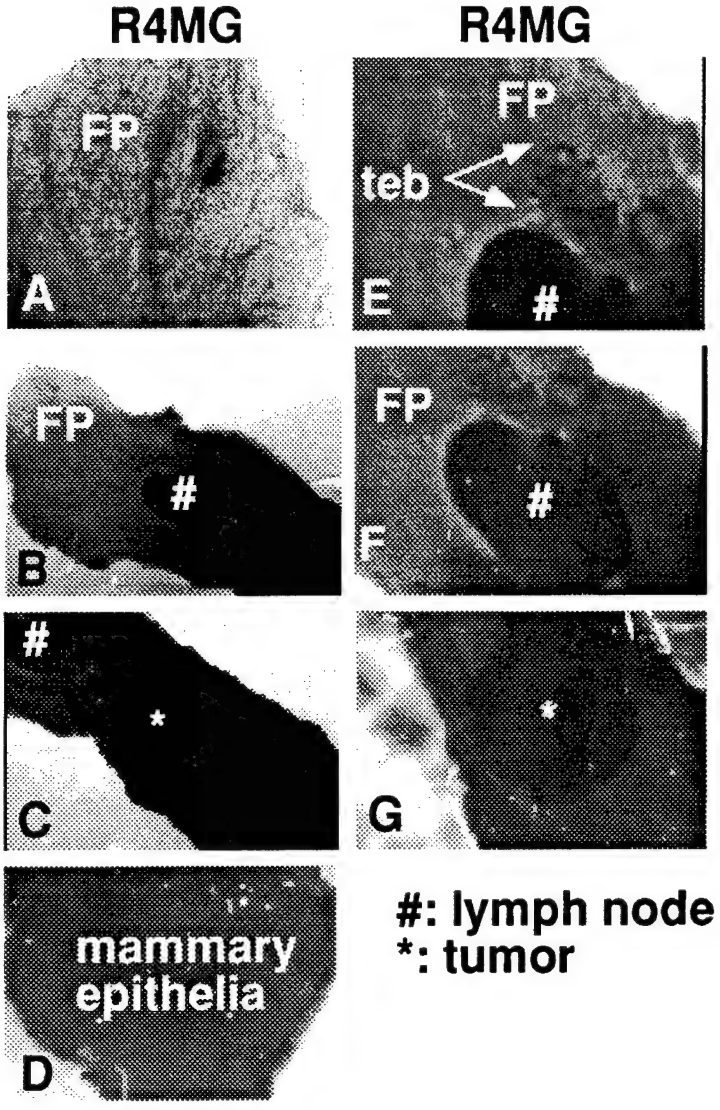


FIGURE 12

Histological analyses of tumor and wholemount sections of the #392 mammary gland before RU486 administration

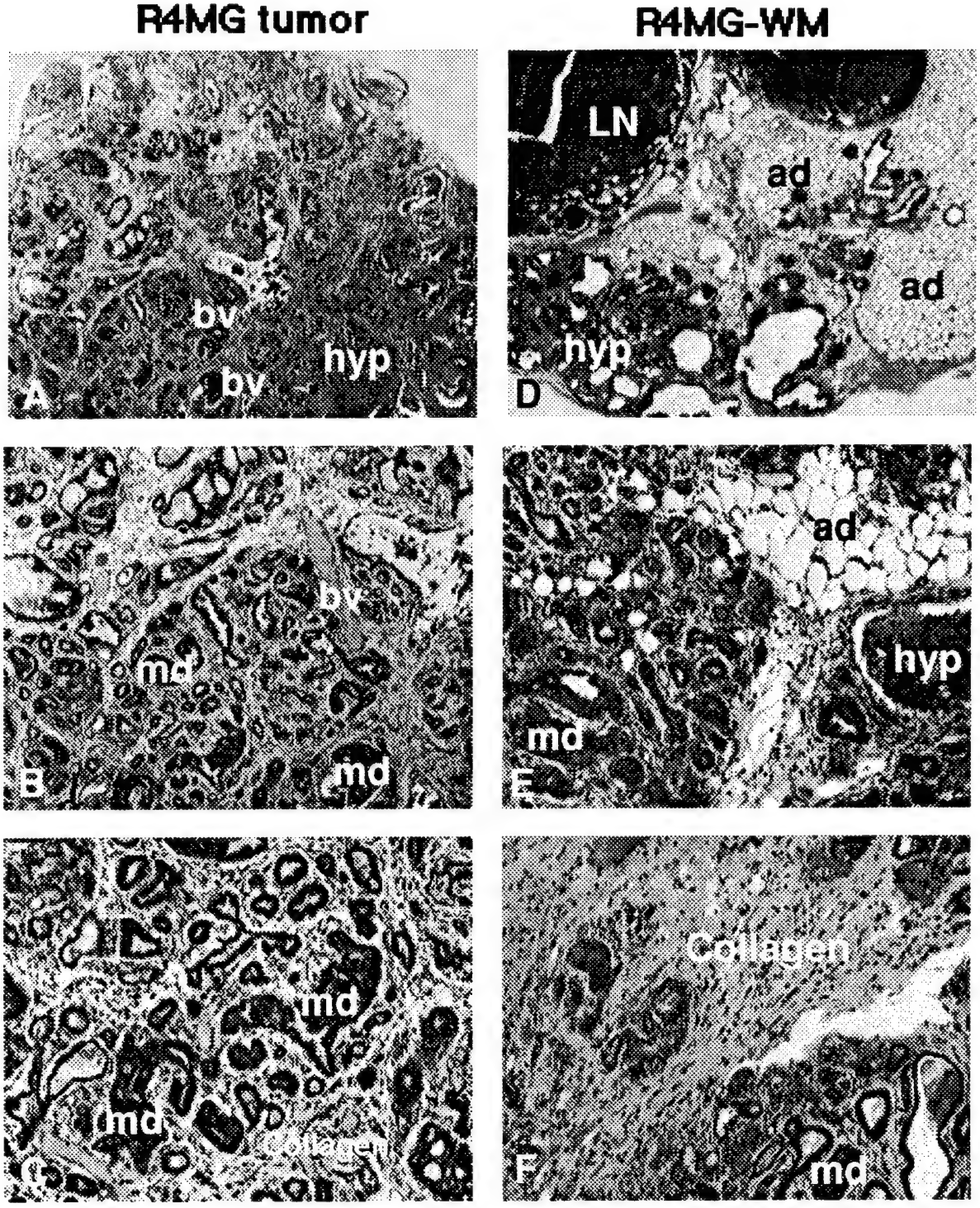


FIGURE 13

Histological analyses of mammary gland outgrowths of the #392 bitransgenic mouse

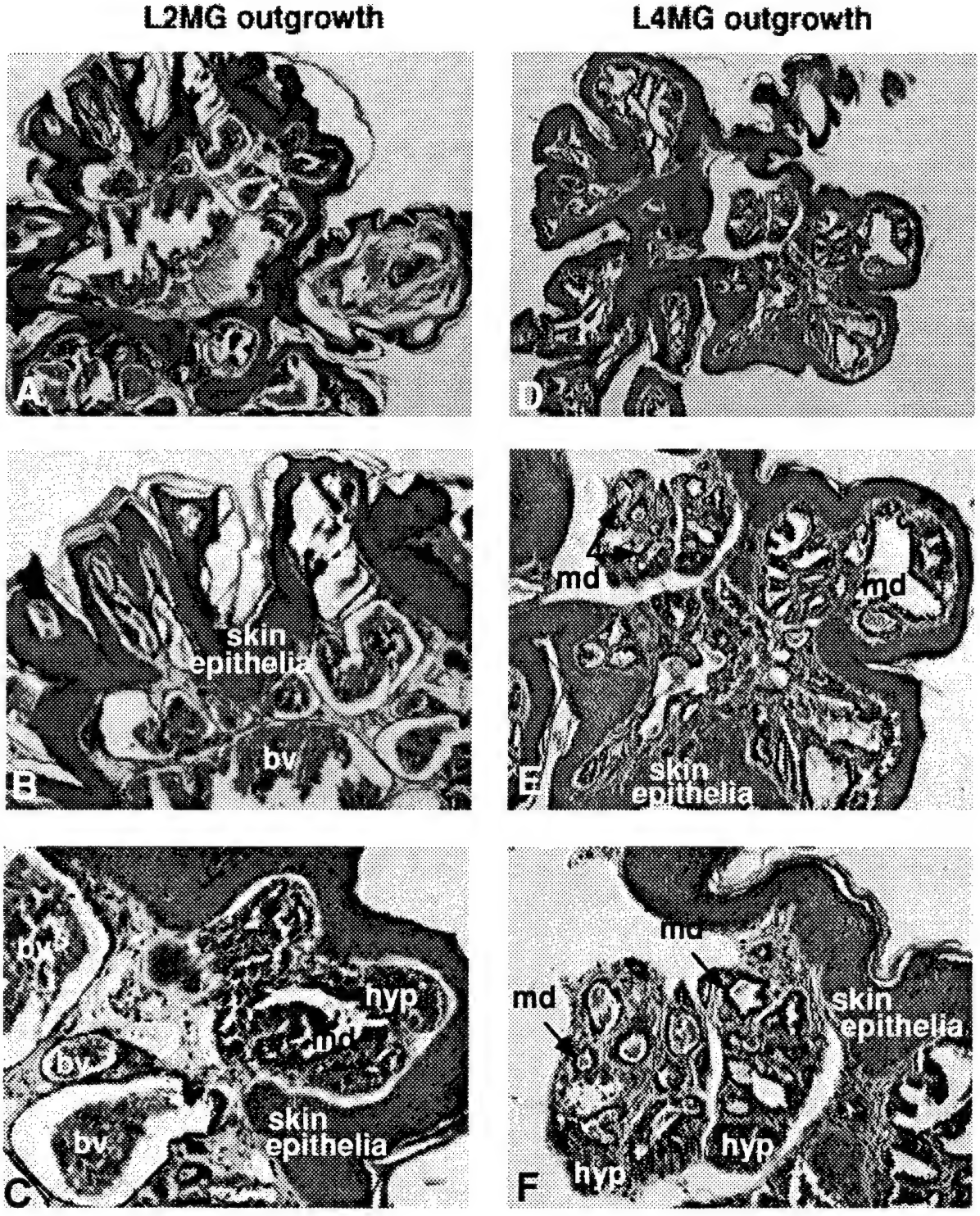


FIGURE 14

Wholemount analyses of mammary glands of #392 bitransgenic mouse after RU486 administration

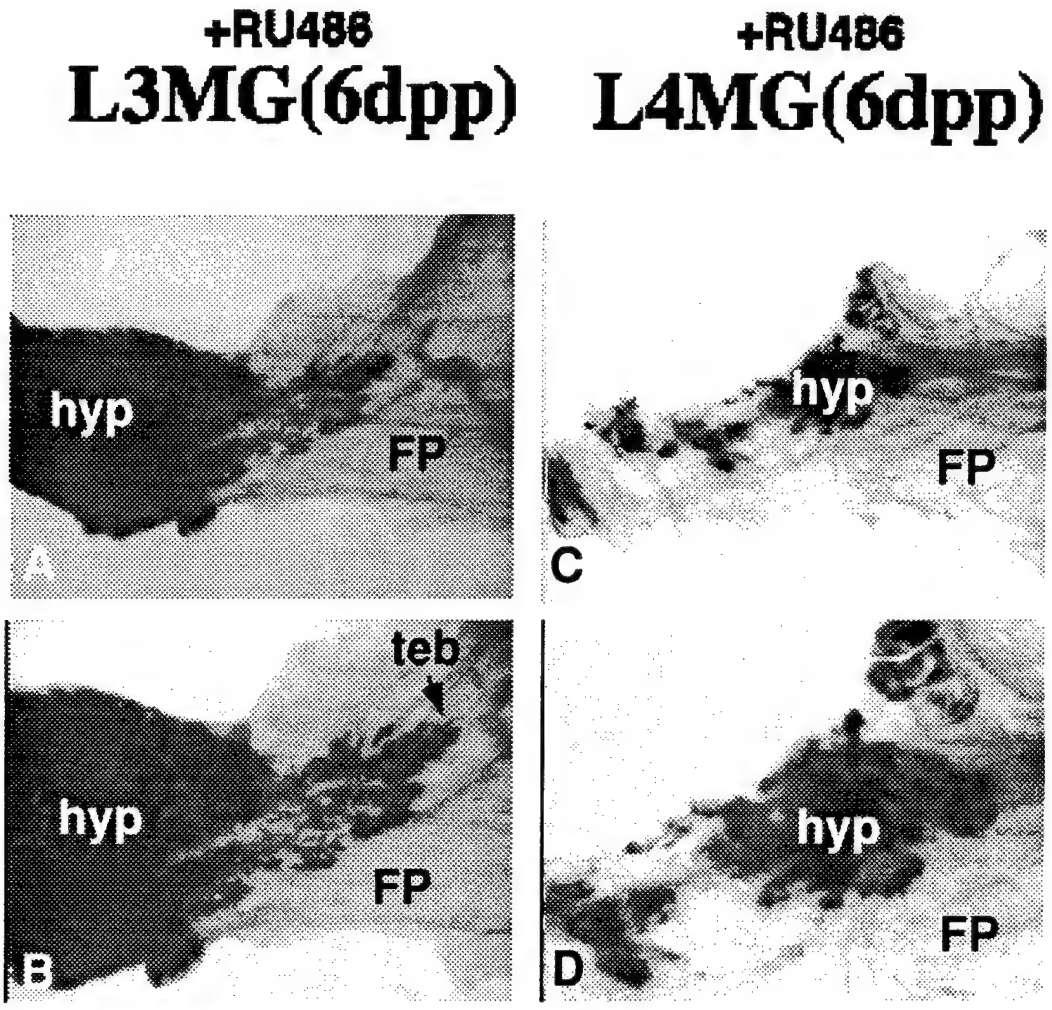


FIGURE 15

Histological analyses of the wholemount sections of #392 bitransgenic mouse mammary glands after RU486 treatment

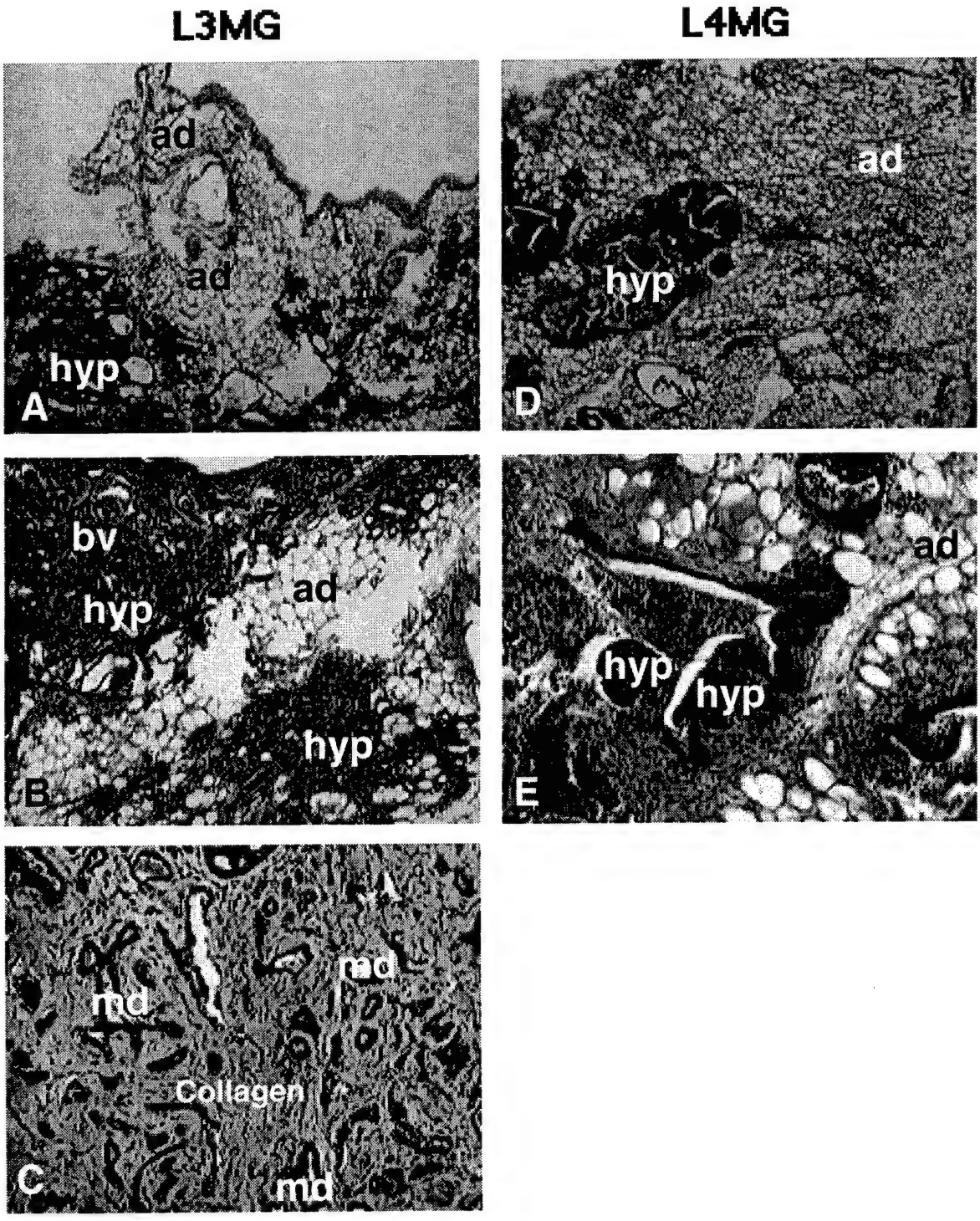


FIGURE 16

Northern analysis of 392 mice tissues

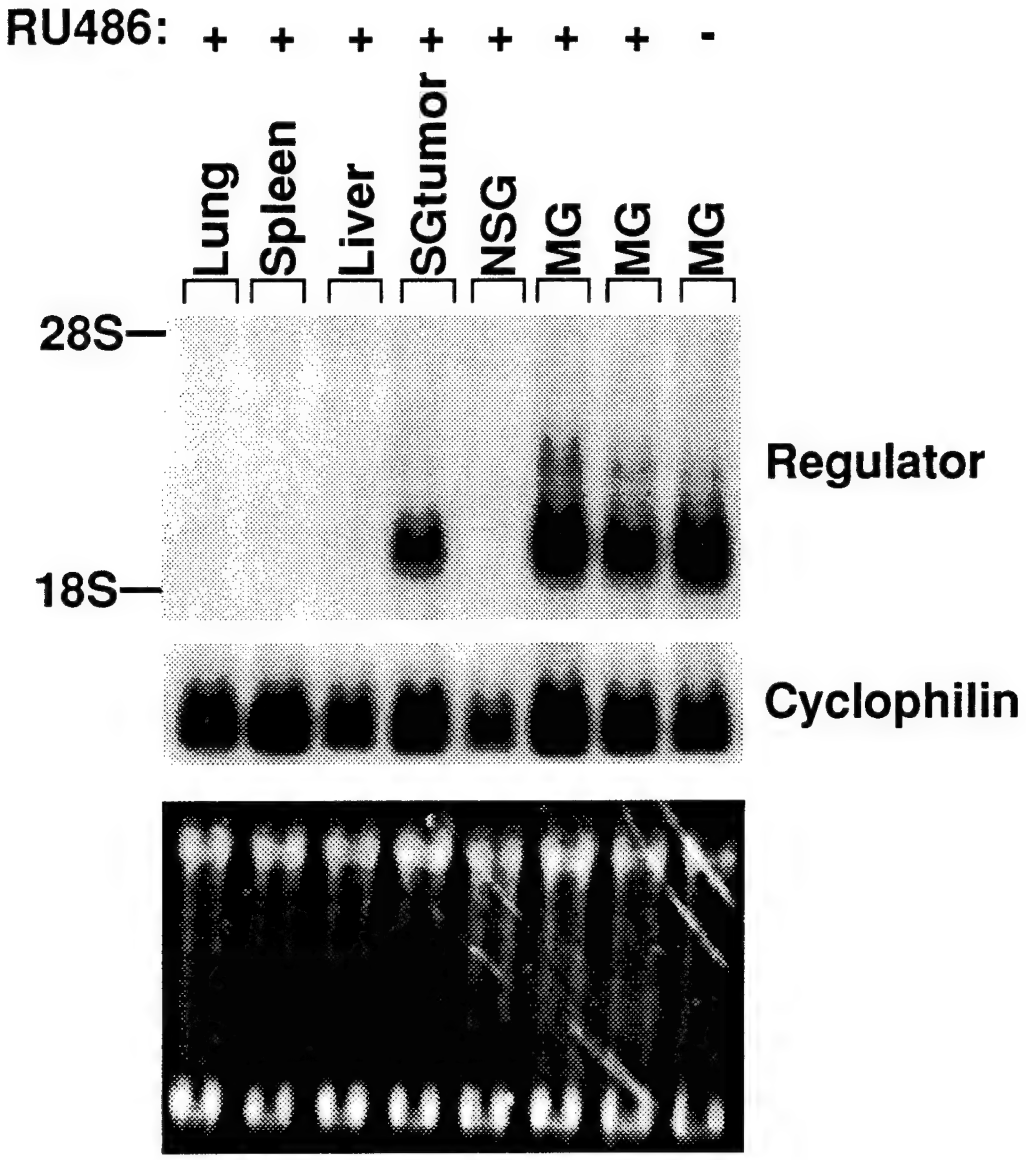


FIGURE 17

Northern analysis of bitransgenic mice tissue RNA for fgf-3/int-2 expression

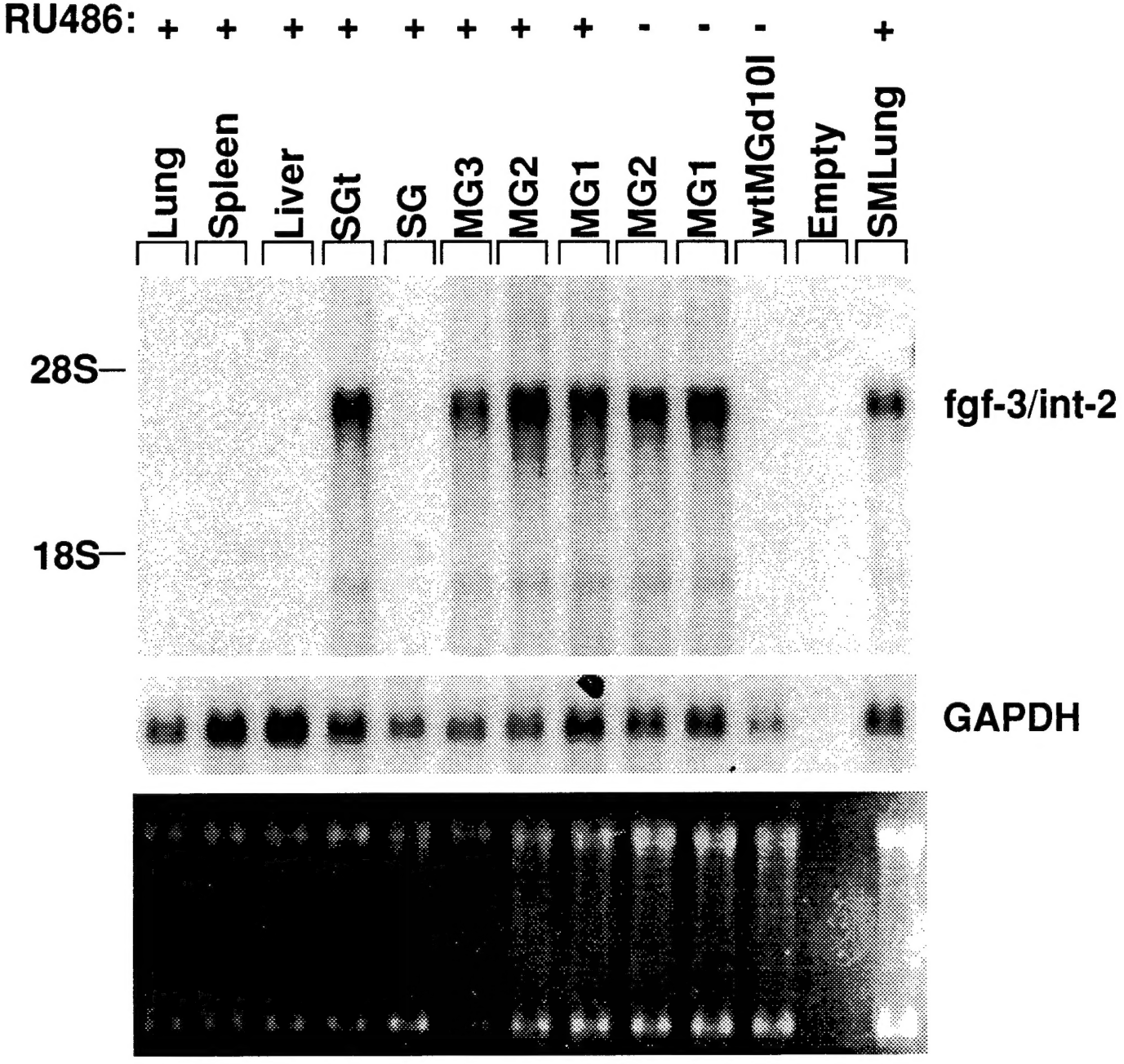


FIGURE 18

Northern analysis of bitransgenic mouse for endogenous C3H MMTV expression

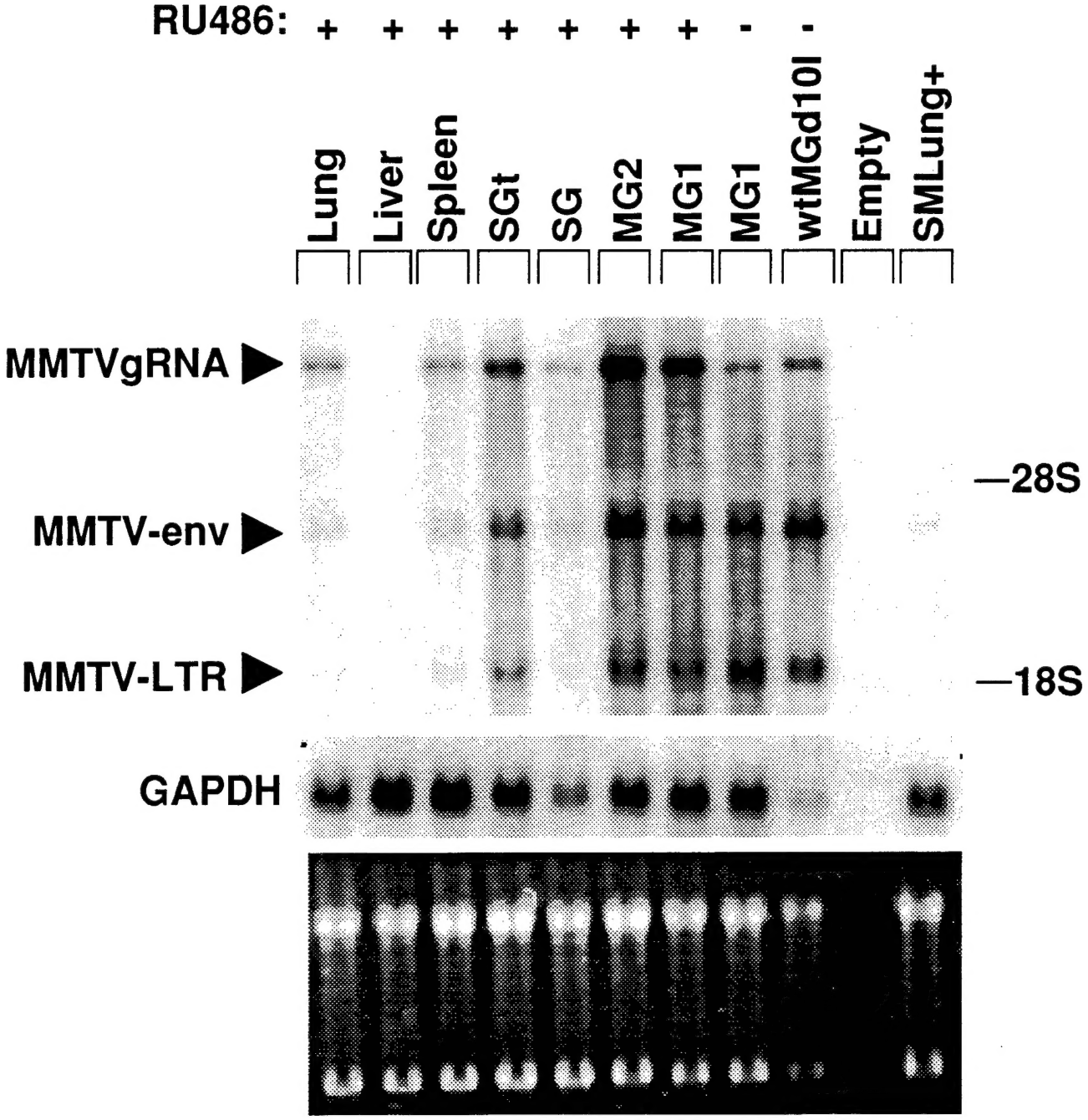


FIGURE 19

Northern analysis of bitransgenic mouse for C3H MMTV env gene expression

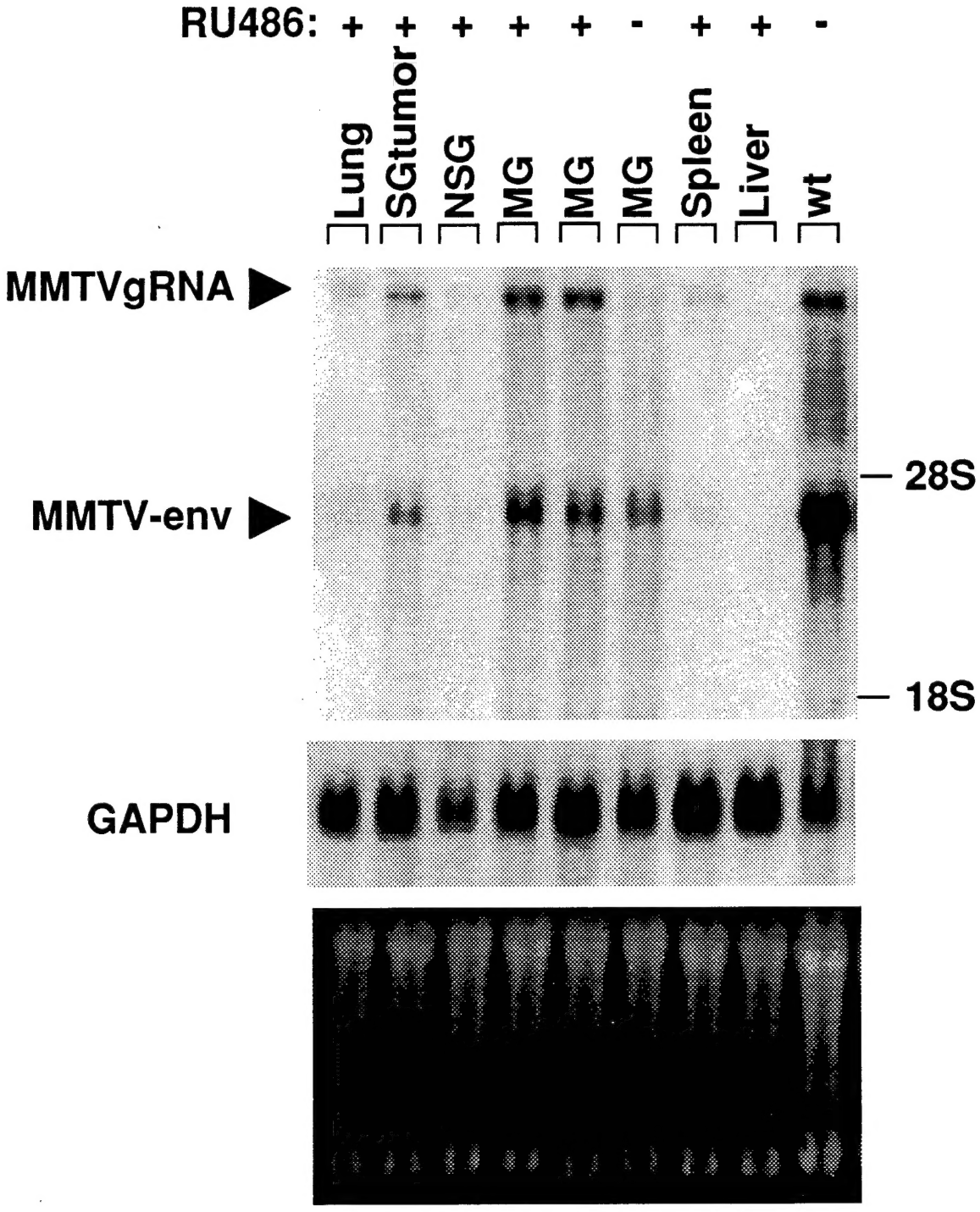


FIGURE 20

Southern analysis of tail DNA of progeny from the HMMB 9478 expressing line

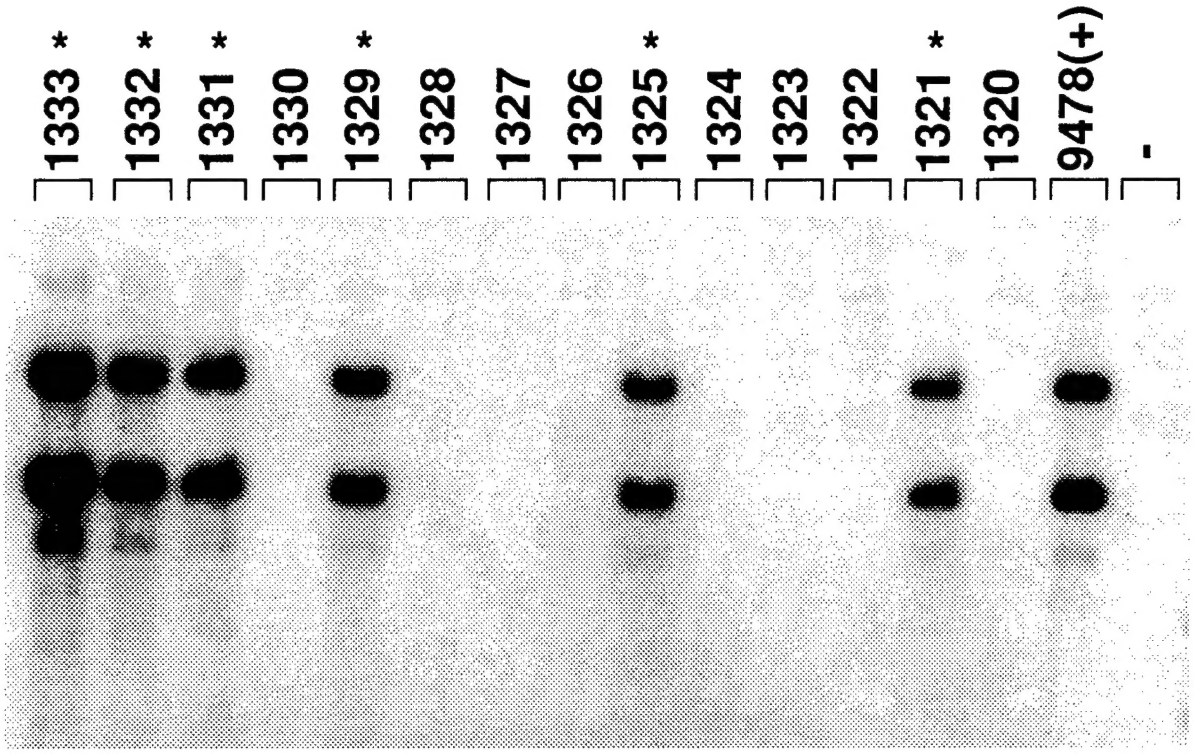


FIGURE 21